EVALUATION OF THE AGE-DEPENDENT ROLE OF NATURAL KILLER T CELLS IN GAMMAHERPESVIRUS INFECTION

by

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ABSTRACT

Epstein-Barr virus (EBV), a human gammaherpesviruses, demonstrates age dependent-pathogenesis. EBV infection in young children is usually asymptomatic, whereas primary EBV infection later in life frequently results in the development of infectious mononucleosis (IM) due to uncontrolled expansion of CD8+ T lymphocytes. Natural killer (NK) T cells are innate T lymphocytes that respond to various pathogens through recognition of lipid antigens presented by CD1d receptors. Previous findings suggested that NKT cells have cytotoxic effects on EBV-infected cell lines. Furthermore, infection with other herpesviruses such as Kaposi’s sarcoma-associated herpesvirus (KSHV) and herpes simplex virus (HSV) can modulate the host immune response to evade NKT cells. To explain the age dependence of EBV pathogenesis, we hypothesized that there is an early window of opportunity to control gammaherpesvirus infection by invariant NKT cells. To test our hypothesis, we infected both neonatal and adult wild type and CD1d knock out (CD1d KO) C57BL/6 mice with murine gammaherpesvirus 68 (MHV-68). Our readout was the quantification of virus copy number by qPCR from genomic DNA extracted from lung and spleen. We report that there was no significant difference in symptoms and viral load between adult and young C57BL/6 and CD1dKO mice. We conclude that NKT cells do not have a major role in the control of gammaherpesvirus infection. The inability of NKT cells to control MHV-68 infection may be due to virus-mediated downregulation of CD1d expression on B cells.
PREFACE

This dissertation is independent work by the author S. Cho. All work from this dissertation is unpublished and original.
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ABBREVIATIONS

EBV - Epstein-Barr virus

CD1dKO – CD1d deficient mice

DCs – dendritic cells

NKT cells – invariant natural killer T cells

MOI - multiplicity of infection

MHV-68 – murine gammaherpesvirus 68

IM- infectious mononucleosis

TLR – toll like receptor

SAP – SLAM associated protein

FOXP3 – forkhead box P3

KSHV - Kaposi’s sarcoma-associated herpesvirus

MIR - modulator of immune recognition

PTLD - post transplant lymphoproliferative disease

IFN-γ – Interferon gamma

TRAIL - tumor necrosis factor-related apoptosis-inducing ligand

TNF – tumor necrosis factor

VZV - varicella-zoster virus

YFP - yellow fluorescent protein

PBMC - peripheral blood mononuclear cells

PFU – plaque forming unit
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1 INTRODUCTION

1.1 GAMMAHERPESVIRUS

1.1.1 EARLY AND LATE EBV EXPOSURE

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects over 90% of the human population. However, EBV rarely causes severe disease and is able to persist for the lifetime of the infected individual due to sophisticated immune evasion strategies (Ebell, 2004). EBV is transmitted primarily via saliva, and the age of acquisition varies widely between regions (Haahr, et al. 2004). Primary EBV infection is nearly universal during early childhood in developing countries, whereas in well developed countries primary EBV infection frequently occurs in adolescents and young adults (Haahr, et al. 2004). It has been suggested that infants from developing countries have higher chance of acquiring EBV due to higher number of siblings in the family and exposure to EBV shedding in saliva by siblings (Rostgaard, et al. 2014). In addition, cultural behaviours, including premastification of food may contribute to higher risk of EBV infection during infancy in developing countries.

In highly developed countries and among people with high socioeconomic status, EBV infection is frequently delayed until adolescence or early adulthood (Gongidi, et al. 2013). While EBV infection in early childhood is usually asymptomatic, later acquisition holds a substantial incidence and risk of infectious mononucleosis (IM) (Ebell, 2004). Symptoms of infectious mononucleosis include sore throat, malaise, and fever. These symptoms typically resolve within weeks, but may last several months and are a major cause of missed school and work. Antivirals or corticosteroids have not been shown to mitigate the course of IM. In addition, EBV infection is associated with several malignancies
of global health importance, including B cell lymphomas, nasopharyngeal carcinoma, and gastric carcinoma (Cohen 2000). As a result, there is a strong impetus to develop a vaccine to prevent EBV-related IM and cancer (Cohen 2000).

1.1.2 EBV PATHOGENESIS

EBV is an enveloped, double-stranded DNA virus. EBV is primarily transmitted through saliva, and the virus shows a specific cell tropism toward B lymphocytes (Thorley-Lawson, et al. 2013). Like all herpesviruses, the EBV life cycle is characterized by both lytic and latent programs of viral gene expression. When virus particles are transmitted to the mouth, the initial virus replication may occur in oral epithelial cells, with production of infectious virions and amplification of the infection. After the lytic phase is completed, at some point, the virus infiltrates oral lymphoid tissue such as tonsils, and infects naïve B cells. Viral entry into naïve B cells requires binding of the viral glycoprotein gp350 to the CD21 receptor on the B cell surface (Busse, et al. 2010). One intriguing possibility is the use of oropharyngeal B cells as a transfer vehicle for epithelial cell infection (Shannon-Lowe, et al. 2006). It has been observed that most EBV virions were bound to B cell surface without internalization, and increased epithelial cell infection efficiency by $10^3$ to $10^4$ fold in vitro (Shannon-Lowe, et al. 2006). After lytic replication of the virus, Infected naïve B cells undergo germinal center (GC) reaction, and the virus expresses full panel of latency associated gene products which include Epstein-Barr nuclear antigens 1 and 2 (EBNA), viral membrane proteins (LMP-1 and 2A, 2B), and non translated viral RNA (EBER) (Chau, et al. 2006). Released infected B cells from germinal center become resting memory B cells,
which enter blood circulation. In infected resting memory B cells, the virus turns off the majority of viral genes other than latency-associated genes. During latency, Epstein-Barr nuclear antigen 1 (EBNA1) attaches the viral genome to host chromosomes during B cell division to allow the viral genome to be passed to daughter cells (Hung, et al. 2001). Viral cis element (OriP) contains EBNA1 binding sequence, and OriP is also required for episome maintenance (Yates, et al. 1984). Infected B cells can migrate back to oral lymphoid tissues, and continuous EBV shedding will occur in healthy adults when infected B cells release virions to oral epithelial cells (Hadinoto, et al. 2009).

1.1.3 MHV-68 PATHOGENESIS

Murine gammaherpesvirus 68 (MHV-68) is a small mouse model commonly used to study human gammaherpesvirus pathogenesis since MHV-68 can be propagated in vitro while propagation of EBV in vitro is difficult. EBV and MHV-68 are closely related gammaherpesvirus, and share similar pathogenesis, which includes lytic and latent phase of lifecycle, and the ability to cause splenomegaly. Also, both EBV and MHV-68 require reactivation transcription activator (RTA) during reactivation from latently infected cells, which is well conserved among different gammaherpesvirus (Liu, et al. 2000). During latency, EBV requires EBNA 1 to maintain circular viral DNA as an episome while MHV-68 requires ORF73. These features allow investigation of MHV-68 viral gene function from both lytic and latent life cycle.

MHV-68 was originally isolated from wild Bank Vole from Slovakia, and the virus readily infects laboratory rodents (Nash, et al. 2001). The virus life cycle involves lytic
replication in the lung during acute infection, which is followed by latency in memory B cells found in the spleen. Following intranasal injection of MHV-68, virus replication in the lung is cleared between day 10 and 12 (Barton, et al. 2011). During acute infection, infected B cells from the lung enter the circulation, and latent virus can be detected in the spleen beginning at 10 days, followed by peak viral copy number in the spleen at 14 days (Hughes, et al. 2010). On day 14 after infection, about 12% germinal center B cells are positive for MHV-68 viral DNA (Flano, et al. 2002).

The transition from acute to latent viral cycle requires B cells. While latent viral DNA can be detected in the lung after acute infection, viral DNA is not detected in the spleen of B cell deficient mice during latent infection (Usherwood, et al. 1996). Furthermore, splenomegaly is not observed in latently infected B cell deficient mice, which suggests that the expansion of latently infected cells require B cells (Usherwood, et al. 1996). Although it is unclear whether latency in the spleen requires infected B cells from the lung or direct infection of B cells in the spleen from cell free virus, the virus requires germinal center B cells to establish and maintain latency in the spleen (Flano, et al. 2000).

1.1.4 EBV IMMUNE CONTROL

CD4+ and CD8+ T cells, and viral antigen presentation by B cells, are critical for control of EBV infection. During primary EBV infection, CD8+ T cells, which are specific to lytic and latent EBV epitopes, will expand vigorously. Callan et al. showed that circulating lytic EBV epitope specific CD8+ T cells are significantly larger in number then latent epitope (Callan, et al. 1998). After primary infection, both lytic and latent specific CD8+ T cells had similar
frequency in the peripheral blood, and these cells could be detected even 3 - 37 months post-infection (Callan, et al. 1998).

In healthy adults, CD8\(^+\) T cells can eliminate infected lymphoblasts, which expand infected IgD\(^-\) and CD23\(^-\) memory B cell populations (Babcock, et al. 1999). When host immune system is suppressed due to post transplant lymphoproliferative disease (PTLD), there was a significant increase in infected resting memory B cells (Babcock, et al. 1999). Also, peripheral B cells from PTLD patients contained linear viral genome whereas healthy individuals had a lower number of infected resting memory B cells and latent circular viral genome (Babcock, et al. 1999). Therefore, a decreased cytotoxic T cell response allowed infected lymphoblasts to escape from CD8\(^+\) T cell mediated cytotoxicity, and produced more infected resting memory B cells. Furthermore, infected memory B cells in peripheral blood were lytic from PTLD patients since the viral genome was linear (Babcock, et al. 1999).

In further support for the important role of T cells to control EBV infection, the use of EBNA1 specific T cells to treat severe EBV infection in PTLD patients could decrease symptoms of EBV related complications (Icheva, et al. 2013). Icheva et al. isolated EBNA1 specific and interferon gamma (IFN-\(\gamma\)) expressing T cells from whole blood, and 10 PTLD patients received EBV specific T cells. The virus specific T cells took 3 to 28 days to expand, and there was detectable expansion of EBV specific T cells from eight out of ten patients which led to decrease in EBV load by more than 1 log (Icheva, et al. 2013). Also, transfusion of EBV latency (LMP-2) specific cytotoxic T lymphocytes (CTL) to nasopharyngeal carcinoma patients showed decreased size of tumor and 1 log decrease in EBV viral load from the plasma (Secondino, et al. 2012).
Viral antigen presentation on B cells plays an important role in controlling gammaherpesvirus infection. Young patients who have X-linked lymphoproliferative (XLP) disease are very susceptible to EBV infection while EBV infection in young patients is asymptomatic (Tatsumi and Purtilo 1986). XLP patients have a rare mutation in the SH2D1A gene, which encodes SLAM associate protein (SAP), and NKT cell development requires SAP for maturation (Palendira et al. 2011). Palendira et al. investigated the effect of SAP on CD8 T cells and they found that SAP-/- CD8 T cells expressed very low levels of CD107a and that B cells could not interact with SAP-/- CD8 T cells (Palendira, et al. 2011). Consequently, it has been hypothesized that B cell antigen presentation is not efficient in XLP patients, and inefficient B cell antigen presentation may contribute to high mortality rates during EBV infection (Palendira, et al. 2011).

1.1.5 MHV-68 IMMUNE CONTROL

In parallel with EBV immune control, CD8+ and CD4+ T cells play an important role in clearing virus in the lung during the acute phase of MHV-68 infection (Ehtisham, et al. 1993). Depletion of T cell-mediated responses cause high mortality in mice, and the viral titer continuously increases in the lung while wild-type mice can clear the virus infection in the lung after acute infection compared to T cell depleted mice (Ehtisham, et al. 1993).

Gredmark-Russ et al. designed MHV-68 tetramers which are specific to p56 (ssDNA binding protein) and p79 (ribonucleotide reductase) lytic viral epitope, and reported that the number of MHV-68 specific CD8+ T cells peaked on day 6 and day 10 post-infection in the spleen (Gredmark-Russ, et al. 2008). When CD8+ T cells were depleted, infected mice
could not clear the virus in the lung after acute infection (Cardin, et al. 1996). Molloy et al. also reported that the absence of CD4\(^+\) T cells during MHV-68 infection caused IL-10 production in CD8\(^+\) T cells, which suppressed antiviral response (Molloy, et al. 2011). Also, CD4\(^+\) T cell depletion led to a delay in clearance of virus in the lung during the acute phase of infection (Ehtisham, et al. 1993). It is also noteworthy to mention that CD4\(^+\) T cells are required to prevent immune exhaustion of CD8\(^+\) T cells (Cardin, et al. 1996).

Although CD4\(^+\) T cells deficient mice could clear virus infection in the lung at the end of acute infection, the virus titer started to increase in the lung on day 30 during latent infection. As a result, CD4\(^+\) T cells are required for continuous clonal expansion of CD8\(^+\) T cells, which lead to clearance of lytic viral replication in the lung.

When latency is established, MHV-68 limits expression of the majority of viral genes while ORF73 maintains viral DNA as an episome (Fowler, et al. 2003). Also, elevation of IL-10 was observed during the viral latency since increase in IL-10 promotes expansion of B cells, which leads to splenomegaly (Siegel, et al. 2008). While EBV encodes BCRF1, which is an IL-10 homologue, MHV-68 uses M2 viral protein to cause elevation of IL-10, which lead to continuous survival of infected B cells (Siegel, et al. 2008).

1.2 INVARIANT NATURAL KILLER T CELLS (NKT CELLS)

Invariant natural killer T cells (NKT cells) are innate-like T lymphocytes, which unlike conventional T cells, can respond to various types of lipid antigens (Matsuda, et al. 2008). The hallmark of NKT cells is the ability to bridge the innate and adaptive immune systems
due to NKT cells’ ability to release large quantities of Th1-, Th2-, and Th17- type inflammatory cytokines.

Activation of NKT cells require CD1d receptors from antigen presenting cells (APCs) and invariant T cell receptors on NKT cells; together this activation results in Th1-, Th2-, or Th17- type inflammatory cytokine release which depends the specificity of different lipid antigen types. Also, activated NKT cells treated with alpha galactosylceramide (α-GalCer) possess multiple pathways to eliminate target cells by apoptosis, which include perforin, or FAS ligand and FAS interaction, tumor necrosis factor (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptotic pathways (Nieda, et al. 2001). Furthermore, when NKT cells recognize α-GalCer presented by antigen presenting B cells, activated NKT cells can allow B cells to form germinal centers and provide help to deploy an IgG antibody response, which depends on IL-21 and IFN-γ (Vomhof-DeKrey, et al. 2014). In addition, when α-GalCer is presented by DCs, NKT cells license DCs to recruit more CD4+ T cells, which is followed by B cell activation (King, et al. 2012). As a result, plasma cells and more germinal centers can be formed when NKT cells allow recruitment of CD4+ T cells via DCs.

Type 1 and type 2 NKT cells can recognize different lipid antigens (Matsuda, et al. 2008). While type 1 NKT cells can recognize glycolipids such as α-GalCer and isoglobotrihexosylceramide (iGb3), type 2 NKT cells cannot recognize α-GalCer and iGb3 (Godfrey, et al. 2010). However, type 2 NKT cells recognize a wider ranges of lipid antigens, such as sulfatide and small aromatic molecules. Type 1 and type 2 NKT cells are defined by different arrangements of their T cell receptors. Type 1 NKT cells have an invariant T cell
receptor (Vα14- Jα18), whereas type 2 NKT cells can have wider ranges of TCR arrangement (Godfrey, et al. 2010). Type 1 NKT cells can be identified by CD1d tetramer, which binds to an invariant T cell receptors, whereas identification of type 2 NKT cells is difficult due to lack of specific antibody (Godfrey, et al. 2010).

1.2.1 ROLE OF NKT CELLS DURING VIRAL INFECTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) is another human gammaherpesvirus, and is causally-associated with Kaposi’s sarcoma (Ganem 2006). Sanchez et al. used the latently infected lymphoma cell line, BCBL-1 lymphoma cells, and observed the difference between CD1d expression before and after virus reactivation by phorbol ester (Sanchez, et al. 2005). Reactivation could reactivate 5-20% of total BCBL-1 cells, and cells in lytic cycles were identified by virus encoded surface marker K8.1. 4 days after reactivation, there was a significant 10 fold reduction in the level of CD1d on infected and lytic K8.1+ BCBL-1 cell surface compared to latently infected K8.1- cells (Sanchez, et al. 2005).

Hepatoblastoma cells (HepG2) express high level of CD1d on the cell surface, and transfection of HepG2 cells with KSHV lytic viral protein such as viral protein modulator of immune recognition (MIR) also significantly reduced surface expression of CD1d (Sanchez, et al. 2005). It still remain elusive if aggravation of KSHV infection from human immunodeficiency virus (HIV) infected patients is due to depletion of NKT cells or CD4+ T cells since HIV infection causes depletion of both NKT cells and CD4+ T cells (Sandberg, et al. 2002).
The role of NKT cells to control herpes simplex virus 1 (HSV-1) has been well elucidated. HSV infection also causes downregulation of CD1d receptor when macrophages and dendritic cells are infected (Yuan, et al. 2006). While HSV-1 did not directly inhibit CD1d or increase CD1d endocytosis, the virus infection prevented CD1d recycling from endocytosed CD1d to the surface by fusing endocytosed CD1d to lysosomes (Yuan, et al. 2006). Grubor-Bauk et al. showed that CD1d-deficient mice had increased morbidity and impaired clearance of the virus (Grubor-Bauk, et al. 2003).

However, in the mouse model, there is no evidence that NKT cells can control MHV-68 infection in adult mice since viral titer from lung during acute infection was the same between C57BL/6 and CD1d deficient adult mice (Usherwood, et al. 2005). The depletion of NK1.1 by neutralizing antibody did not increase MHV-68 titer in the lung of mice after 2 days post-infection (Usherwood, et al. 2005). NKT cells require IL-15 to develop, and the use of IL-15 deficient mice showed no difference in virus titer between IL-15 deficient and wild-type mice (Usherwood, et al. 2005).

1.2.2 NEONATAL NKT CELLS

Conventional T cells start to develop in the thymus on day 17 and 18 during embryogenesis whereas development of NKT cells is delayed until 7 days after birth in mice (Hammond, et al. 1998). While neonatal mice NKT cells take a longer time to develop, neonatal humans have NKT cells present in the umbilical cord blood. From human umbilical cord, 0.061% cells are Vα24\textsuperscript{+}Vβ\textsuperscript{+} NKT cells, and these NKT cells can be activated and expanded by α-GalCer stimulation in vitro (Kawano, et al. 1999). Neonatal NKT cells express
CD25, which is a marker of recent activation, and these cells do not release IFN-γ and IL-4 during PMA and ionomycin stimulation, although these cytokines could be detected from adult NKT cells from peripheral blood by intracellular staining (D’Andrea, et al. 2000). Based on these observations, maturation of human NKT cells requires a second activation by antigens.

Our understanding of neonatal NKT cells and antigen interaction is still in its germinal stage of investigation. Levy et al. reported that an 11-year-old girl with NKT cell deficiency had disseminated varicella-zoster virus (VZV) infection from the live attenuated vaccine strain of VZV (Levy, et al. 2003). Laboratory testing showed alveolar infiltration and normal VZV-specific lymphocyte proliferation and NK-cell cytotoxicity. However, the patient had fewer NKT cells (<0.3%) from peripheral blood mononuclear cells (PBMC) compared to a healthy control subjects (1-2%), and isolated NKT cells could not respond to α-GalCer.

Olszak et al. used a mouse model to investigate the early interaction between innocuous antigens such as gut microbes and neonatal NKT cells. When germ free adult mice were challenged with oxazolone induced colitis, germ free adult mice had more severe colitis than wild-type adult mice. There was a significant increase in IL-1β and IL-13 from the colon of adult germ free mice, and CD1d neutralizing antibody significantly decreased level of IL-1β, IL-13, and the number of NKT cells in the intestine. Before oxazolone induced colitis challenge, germ free adult mice had significantly higher number of intestinal NKT cells, which were the source of IL-1β, IL-13 and colitis aggravation. Only germ free pups, which were exposed to gut microbes had a significant reduction in symptoms of colitis and reduced NKT cells in the intestine. The result suggests that NKT cells have an early window
of opportunity to interact with antigens to develop immune tolerance, and it is not very clear if the interaction between neonatal NKT cells and innocuous pathogen such as EBV or MHV-68 interaction has a protective or regulatory effect.
2 HYPOTHESIS AND RATIONALE

2.1 HYPOTHESIS

NKT cells can control acute MHV-68 infection in young mice and protect against severe disease.

2.2 SPECIFIC AIMS

1) To compare symptoms of MHV-68 infection between CD1d knock-out and wild-type C57BL/6 pups.
2) To compare the level of MHV-68 replication between CD1d knock-out and wild-type C57BL/6 pups
3) To compare CD1d expression on B cells between infected and uninfected wild-type C57BL/6 mice.

2.3 RATIONALE

Our preliminary hypothesis was supported by Chung et al. since there is evidence that NKT cells can inhibit expansion of EBV infected B cells and eliminate EBV transformed lymphoblastoid cell line (LCL) (Chung, et al. 2013). Chung et al. observed a significant two-fold increase in EBV infected B cells when PBMCs from healthy individuals were incubated without NKT cells for 5 days. Furthermore, inhibition of EBV transformed B cells was CD1d dependent, and induction of CD1d expression on LCL by use of a small molecule AM580 significantly increased cytotoxicity of NKT cells against EBV transformed LCL (Chung, et al.
2013). Also, patients with EBV associated cancer have fewer NKT cells, and the function of these NKT cells is impaired (Yuling, et al. 2009).

The role of NKT cells during early gammaherpesvirus infection is not well studied. It is unknown if NKT cells can provide protection during early gammaherpesvirus infection, although previous findings suggest that NKT cells are not important to control gammaherpesvirus in adults (Usherwood, et al. 2005). Although recent evidence favours NKT cell independent control of MHV-68 infection in adult mice, the function of NKT cells may be age-dependent. In mouse models, NKT cells appear to be important for establishing tolerance against non-pathogenic antigens in early life (Olszak, et al. 2012). Although early NKT cells and gut microbial interaction is not identical to early NKT cells and EBV interaction, we were interested in determining if there is an early window of time for NKT cells to prevent dissemination of MHV-68 since CD4+ and CD8+ T cell response against virus infection is immature in neonatal mice. We hypothesized that early EBV infection is asymptomatic due to the efficient control of MHV-68 by neonatal NKT cells.
3 MATERIAL AND METHODS

3.1 ANIMALS

C57BL/6 and CD1d deficient (CD1d KO) mice on a C57BL/6 background were purchased from Jackson Laboratory (Sacramento, CA, USA). A maximum of five animals were housed in one cage. Two to three month old male and females from C57BL/6 or CD1d KO were crossed to make breeding pairs. Three breeding pairs for both C57BL/6 and CD1d KO mice were prepared which contained one male and two female mice. All uninfected mice were housed in pathogen free conditions at the CFRI animal care unit. Infected animals were housed in the CL2 area within the CFRI animal care facilities. All animals were monitored closely based on MHV-68 infection clinical score. Briefly, infected animals were scored based on hydration, piloerection, and activity on a scale of 0 to 3: 0, no clinical sign; 1, mildly abnormal; 2, abnormal; 3, severe. Clinical score from each category was combined and animals with combined score higher than 5 were euthanized. Infected pups and adult mice were monitored once a day and twice a day on day 5, which is the beginning of lytic viral replication in the lung. All procedures were approved by the Canadian Council on Animal Care (A14-0358).

3.2 VIRUS PREPARATION AND INFECTIONS

MHV-68 and recombinant H2b/YFP MHV-68 (originally acquired from Dr. M. Horwitz) were propagated in Baby Hamster Kidney cells (BHK-21). Virus stocks were titered by plaque assay as described previously (Cho, et al. 2009). When BHK-21 cells reached 50%
confluence, cells were infected with an MOI of 0.01. After two days of infection, unhealthy cells were harvested, and freeze-thawed three times to release membrane associated virus.

Plaque assay was used to determine titer of MHV-68 as described before (Wu, et al. 2000). To determine the titer of virus, Green Monkey Kidney (VERO) Cells were seeded on 6 well plates and seeded 6 well plates were incubated at 37°C for 2 days. Virus stock was serially diluted in ten-fold dilutions with FBS free media up to \(10^{-5}\) (\(10^{-1},10^{-2},10^{-3},10^{-4},10^{-5}\)). Inoculated 6 well plates were incubated for 1 hour at 37°C, and 6 well plates were gently rocked every 15 minutes. Infected vero cells were overlaid with 2% sterile agar media, plates were incubated for 6 days, and stained with 0.25% crystal violet solution.

Before infection, 8 days old pups were anesthetized by isoflurane for 4 to 5 minutes. Anesthetized pups were infected with MHV-68 by intranasal infection in a final volume of 10 μL (1800 pfu). Infected and uninfected pups were co-housed. Adult C57BL/6 and CD1d KO mice were anesthetized by isofluorane for 4 to 5 minutes, and infected with 13000 pfu by intranasal inoculation in a final volume of 40 μl. Adult mice were two to three months old.

3.3 TISSUE PROCESSING FOR GENOMIC DNA EXTRACTION

Infected animals were sacrificed (isoflurane anesthesia followed by cervical dislocation) on day 6 post-infection. Lung tissue was incubated with type IV collagenase (Gibco cat# 17104-019) for 1 hour at 37°C prior to homogenization. Lung and spleen tissues were homogenized with a 5mL syringe plunger and filtered through a 70 μm cell strainer. Homogenized tissue was resuspended to a final volume of 15 mL PBS. Cells from each tissue
were counted, and 8 million cells per sample were used to extract genomic DNA according to manufacturer's protocol (Qiagen blood tissue DNaseasy kit - Cat# 69504). Extracted DNA concentration was measured by nanodrop spectrophotometer (Thermo Scientific).

3.4 qPCR ASSAY TO DETERMINE VIRAL COPY NUMBER

Quantitative polymerase chain reaction (qPCR) was used to measure the copy number of MHV-68 genomes. Each reaction contained 50ng of DNA from lung or spleen. ORF56- specific primers (F: 5'-gtccaggctttcctgtctg-3') (R: 5'-tgcaatgtgtcacaggctta-3') were obtained from Integrated DNA Technologies. Primers and SYBR green master mix (BIO-RAD Cat#172-5211) were used, at the final concentration of 500nM and 1X per reaction respectively. Reactions were run at 95°C for 10 minutes and 45 cycles at 95°C for 30s, 60°C for 1 minute, and 72°C for 10s, which was followed by melting curve analysis. Viral gene copy number was standardized by 10-fold serial dilutions of a known copy number of ORF56 gBlock DNA fragment (Integrated DNA Technologies).

3.5 STAINING AND FLOW CYTOMETRY

Adult wild-type C57BL/6 mice were infected with recombinant H2b/YFP MHV-68 (7200pfu), and spleens were harvested after 16 days post infection. A single cell suspension was prepared from spleen, and 2 million cells were harvested for staining. Cells were incubated with Fc block (eBiosciences cat# 14-0161-82) at room temperature for 5 minutes. After incubation, cells were stained with cell surface markers in FACS buffer (2% FBS and 0.2% EDTA in PBS) for 30 minutes on ice. Stained cells were washed with FACS buffer twice. Cell surface markers to identify infected B cells were CD19 (eBio 1D3), CD1d (eBio 1B1), CD3
(eBio 17A2), 7AAD (eBio Cat# 00-6993-50). Antibodies and viability dye were purchased from eBiosciences. Stained samples were analyzed by using a FACS LSR II (BD Biosciences) and acquired data were analyzed with FlowJo software (Tree Star, Inc).

3.6 STATISTICAL ANALYSIS

GraphPad Prism 5.0 software was used to create tissue weight and viral copy number graphs. Nonparametric test (Mann-Whitney $u$-test) was used to compare tissue weight between pups and adult mice. Viral copy numbers were displayed in logarithmic scale with means and standard error of the means (SEMs), and Student $t$-test was used to calculate $p$ values. $P$ values < 0.05 were considered significant.
4 RESULTS

4.1 EFFECT OF MHV-68 INFECTION ON C57BL/6 AND CD1d KO PUPS AND ADULTS

When wild-type C57BL/6 and CD1d KO pups were infected with MHV-68, there was no clear difference in symptoms. Between day 1 and day 5 post-infection, there were no sign of MHV-68 infection (Figure 1). On day 6 post-infection, both C57BL/6 and CD1d KO pups showed signs of discomfort, which included inactivity and mild piloerection. However, two CD1d KO pups were moribund, and one CD1d KO pup showed signs of rapid breathing and hunched posture. Uninfected pups from C57BL/6 and CD1d KO litter were inactive because they preferred to cluster together when infected pups were sick. Furthermore, there was no significant difference in weight loss due to MHV-68 infection (Figure 2). All infected and uninfected pups gradually gained weight as they were maturing.

We observed the symptoms of MHV-68 infection in adult C57BL/6 and CD1d KO mice to confirm that the control of MHV-68 infection is NKT cell independent. In line with previous observation, there was no significant difference in symptoms between adult C57BL/6 mice and CD1d KO mice. Two to three month old adult C57BL/6 and CD1d KO mice were infected with 13000pfu, and infected mice were monitored daily for weight loss and symptoms for 6 days until mice were euthanized. When adult mice were infected, there were no visible symptoms such as inactivity and piloerection.

All infected adult mice were healthy, and there was no noticeable behavior difference between uninfected and infected mice. Body weight of infected animals remained constant until the end of experiment (Figure 3). Overall, there were no moribund mice until infected mice were euthanized.
Figure 1. No significant difference in visible symptoms between infected C57BL/6 and CD1d KO pups. C57BL/6 and CD1d KO pups were infected with MHV-68 (1800 pfu). Infected pups were carefully monitored and scored based on activity, posture, and gait. Data shown as mean disease score. 8 infected wild-type C57BL/6, 7 uninfected CD1d KO, 3 uninfected CD1d KO and 4 uninfected C57BL/6 pups were used for each data point.
Figure 2. No significant weight difference between infected C57BL/6 and CD1d KO pups.

Body weight was monitored after MHV-68 infection (day 0) and up to day 6 post-infection. 8 infected wild-type C57BL/6, 7 infected CD1d KO, 3 uninfected CD1d KO and 4 uninfected C57BL/6 pups were used for each data point. Data shown as means and bars represent SEMs (standard error from the means).
Figure 3. No significant weight difference between C57BL/6 and CD1d KO adult mice after MHV-68 infection. Body weight was monitored after MHV-68 infection (day 0) and up to day 6 post-infection. 6 infected and 3 uninfected CD1d KO and wild-type C57BL/6 adult mice were used for each data point. Data shown as % mean weight change and bars represent SEMs (standard error from the means).
4.2 EFFECT OF MHV-68 INFECTION ON LUNG AND SPLEEN FROM INFECTED PUPS AND ADULTS

MHV-68 active viral replication in the lung was detectable on 6 days post-infection. When lungs were harvested, there was increased tissue weight when CD1d KO pups were infected with the virus although the difference was not statistically significant (Mann-Whitney test p=0.1) (Figure 4). Both C57BL/6 and CD1d KO infected pups also had larger and heavier spleen than uninfected pups although the difference was not statistically significant (Mann-Whitney test p=0.07). There was no difference in size and weight of spleen between infected C57BL/6 and CD1d KO pups (Mann-Whitney test p=0.6126) (Figure 5).

When lung tissue was harvested from adult mice, there was no significant difference in size and tissue weight between C57BL/6 and CD1dKO infected adult mice (Figure 6). Also, the size and weight of spleen between infected C57BL/6 and CD1dKO adult mice were not significantly different (Figure 7).
Figure 4. Increase in lung weight after MHV-68 infection, but no statistically significant increase in lung weight between C57BL/6 and CD1d KO pups. Lungs were harvested from infected and uninfected pups on day 6 post-infection, and harvested tissue was weighted. 8 infected wild-type C57BL/6, 7 infected CD1d KO, 4 uninfected C57BL/6, and 3 infected CD1d KO pups were used. Data are shown as mean and bar represents SEM (standard error from the mean). Mann-Whitney test was used to analyze data. Calculated p value from infected C57BL/6 and CD1dKO pup was 0.1.
Figure 5. No significant difference in spleen weight after MHV-68 infection between C57BL/6 and CD1d KO pups. Spleens were harvested from infected and uninfected animal on day 6 post-infection, and harvested tissue was weighted. 8 infected wild-type C57BL/6, 7 infected CD1d KO, 4 uninfected C57BL/6, and 3 infected CD1d KO pups were used. Data are shown as mean and bar represents SEM (standard error from the mean). Mann-Whitney test was used to analyze data. Calculated p value from spleens of infected C57BL/6 and CD1dKO pup was 0.6.
Figure 6. No significant change in weight of harvested adult lung tissue 6 days post-infection. Adult mice were infected with 13000pfu and harvested lung tissue was weighted. 6 infected and 3 uninfected mice were used for C57BL/6 and CD1d KO group. Bar represents standard error from the mean. Mann-Whitney test was used to analyze data. Calculated p value from infected and uninfected C57BL/6 and CD1dKO adult lungs was 0.6 and 0.1 respectively.
Figure 7. No difference in weight of harvested spleen from adult mice 6 days post-infection. Adult mice were infected with 13000pfu and harvested spleen tissue was weighted. 6 infected and 3 uninfected mice were used for C57BL/6 and CD1d KO group. Bar represents standard error mean. Mann-Whitney test was used to analyze data. Calculated p values from infected and uninfected adult C57BL/6 and CD1dKO spleens were 0.3 and 0.1 respectively.
4.3 QUANTIFICATION OF VIRAL COPY NUMBER IN C57BL/6 and CD1d KO PUPS AND ADULTS

We next investigated if C57BL/6 and CD1d KO pups had different levels of viral replication as assessed by viral genome copy number. Harvested organs on day 6 post-infection were processed and total DNA extracted from infected C57BL/6 and CD1d KO pups was measured by qPCR. There was no significant difference in virus copy number between C57BL/6 and CD1d KO pups in the lung (Student's t test p=0.5) (Figure 8). Although CD1d KO pups had higher virus copy number in spleen, the difference was less then 10-fold.

Total DNA was extracted from lung and spleen of infected adult mice, and viral copy number from 100ng of DNA was quantified by using qPCR against ORF56. Both C57BL/6 and CD1dKO infected adult mice had significantly lower (2 log difference) viral copy number than infected pups in the spleen and the lung (Figure 9). However, there was no significant difference in viral copy number between C57BL/6 and CD1dKO adult mice in the lung and spleen (Figure 10). Although ORF56 viral gene was detected from spleens of adult mice and pups, there was no actively replicating virus.
Figure 8. No significant difference in viral copy number between infected C57BL/6 and CD1d KO pups. 8 day old pups were infected with 1800 pfu, and after 6 days post-infection, total extracted DNA (50ng) from spleen and lung were used for qPCR to detect ORF56. 8 infected wild-type C57BL/6 and 7 CD1d KO pups were analyzed. Bars represent standard error means. Data were analyzed by Student’s t test, and calculated p value from infected C57BL/6 and CD1dKO lungs and spleen were 0.5 and 0.06 respectively.
Figure 9. Adult mice had significant higher viral load compared to pups in the lung and spleen. 8 C57BL/6 pups were infected with 1800 pfu while 6 C57BL/6 adult mice received 13000pfu. Total DNA was extracted from lung and spleen 6 days post-infection. Viral DNA (ORF56) was detected from 100ng of genomic DNA by qPCR. Data were analyzed by Student’s t test and bars represent standard error of the means. Calculated p value from infected C57BL/6 lungs and spleens were 0.01 and 0.04 respectively.
Figure 10. No significant difference in viral copy number between infected C57BL/6 and CD1d KO adult mice. Two to three month old mice were infected with 13000 pfu, and after 6 days post-infection, extracted DNA from spleen and lung were used for qPCR to detect ORF56. Data were analyzed by Student’s t test. Calculated p value from infected C57BL/6 and CD1d KO lungs and spleens were 0.7463 and 0.2899 respectively.
4.4 DOWNREGULATION OF CD1d RECEPTORS ON INFECTED B CELLS

Although downregulation of CD1d receptors on human gammaherpesvirus infected B cells is well described, it is not clear if MHV-68 infection in B cells causes downregulation of CD1d. To answer the question, three adult C57BL/6 mice were infected with 7200 pfu of recombinant H2b/YFP MHV-68 by intranasal inoculation. After 16 days, spleens from infected mice were processed as single cell suspensions, and harvested cells were stained with surface markers such as CD19 and CD3 to differentiate B cells from splenocytes. Recombinant H2b/YFP MHV-68 express yellow fluorescent protein (YFP), and YFP expressing cassette is located between ORF27 and ORF29b, which is driven by the CMV promoter (Collins and Speck, 2012). Infected B cells with recombinant H2b/YFP MHV-68 will express YFP in the nucleus, and conjugation of YFP with histone molecule H2b allows stable YFP expression during viral latency in the spleen (Collins and Speck, 2012).

0.32% of CD19⁺ CD3⁻ B cells from one of the infected mice was positive for YFP signal, and virus infected YFP⁺ B cells were analyzed for CD1d expression (Figure 11). We observed a significant decrease in CD1d receptor on the surface of infected YFP⁺ B cells when adult mice were infected with recombinant H2b/YFP MHV-68 after day 16 post-infection (Figure 12) (Student's t test p=0.004).
Figure 11. Detection of YFP⁺ splenocytes and downregulation of CD1d receptors on infected YFP⁺ and YFP⁻ B cells. Adult mice were infected with recombinant H2b/YFP MHV-68 and splenocytes were harvested and stained on day 16 post infection (n=3). CD19⁺ CD3⁻ B cells were gated and infected YFP⁺ B cells were visualized on panel A. Panel B shows level of CD1d expression on infected YFP⁺ B cells (red) which is overlaid with infected YFP⁻ B cells (green), uninfected C57BL/6 splenocytes (blue n=2), and uninfected CD1d KO splenocytes (black).
Figure 12. Significant reduction of CD1d median fluorescence intensity on infected YFP+ B cells. Adult mice were infected with recombinant H2b/YFP MHV-68 and splenocytes were harvested and stained after 16 days post infection (n=3). Bar represents standard error of the mean. A Student's t test was used to calculate p value comparing YFP+ B cells and uninfected B cells (p=0.004).
5 DISCUSSION AND CONCLUSIONS

5.1 EARLY MHV-68 INFECTION BETWEEN C57BL/6 AND CD1d KO PUPS

Understanding the immune mechanisms that determine whether primary EBV infection is asymptomatic or results in IM could have a major public health impact on patients in developed counties, and for EBV-related cancers worldwide. We are surprisingly ignorant of neonatal immune response to innocuous pathogens such as EBV. We used MHV-68 as a mouse model for EBV infection to investigate if neonatal NKT cells can prevent dissemination of MHV-68 during in neonatal mice.

Although we speculated that CD1d KO pups would have a higher viral load and aggravated symptoms due to the lack of functional NKT cells, we did not observe significant difference in viral gene copy number between C57BL/6 and CD1d KO pups. Furthermore, on day 6 post-infection, there was no visible difference in symptoms even though 3 CD1d KO pups were moribund. When CD1d KO infected pups were infected, CD1d KO pups had increased lung weight although the increase in lung weight was not statistically significant. It is possible that CD1d KO pups had increase in lung weight compared to infected wild-type pups due to inflammation and infiltration of body fluid. It is possible that NKT cells can play regulatory role in controlling inflammation, which can prevent epithelial cell damage and fluid infiltration into inflamed lung. Based on viral copy number and symptoms, our results show that NKT cells did not have a statistically significant role in controlling MHV-68 infection.

NKT cells use invariant T cell receptors to recognize lipid antigens presented by CD1d receptors on antigen presenting cells. It is not very well understood if viral lipid antigens are
presented on CD1d receptors. Furthermore, there is the possibility that NKT cells are activated without CD1d receptors during virus infections (Juno, et al. 2012). As a result, it is possible that there was no significant difference in viral load between C57BL/6 and CD1d KO pups because NKT cells could be activated by CD1d independent manner.

Without CD1d receptors, NKT cells could be activated when toll like receptors (TLRs) from dendritic cells (DCs) were engaged, which leads to production of IFN-γ. Paget et al. reported that CD1d deficient DCs were not able to activate NKT cells when α-GalCer was incubated, and IFN-γ release from NKT cells was decreased by 70% (Paget, et al. 2007). Nevertheless, these CD1d deficient DCs could activate NKT cells when TLR ligands such as CpG oligodeoxynucleotides (CpG ODN) and LPS were present, and CD1d independent activation mechanism required type I interferon, not IL-12. Since TLR-9 recognize MHV-68 viral DNA, MHV-68 infection may activate NKT cells in a CD1d-independent manner (Guggemoos, et al. 2008).

It is also possible that both C57BL/6 and CD1d KO mice had no difference in symptoms and viral load because lytic and latent MHV-68 infection could downregulate CD1d expression of infected cells. As a result, infected C57BL/6 mice had downregulated CD1d expression in infected cells, which lead to similar viral load between C57BL/6 and CD1d KO mice.

5.2 DOWNREGULATION OF CD1d ON INFECTED B CELLS

We next addressed the possibility that MHV-68 infection downregulated CD1d expression on infected cells. When adult wild-type C57BL/6 mice were infected with
recombinant H2b/YFP MHV-68, we observed a statistically significant reduction of CD1d receptors on infected YFP+ B cells in adult C57BL/6 mice 16 days after infection. It remains as speculation if CD1d downregulation on infected B cells 16 days post infection is biologically significant. For example, while the latently infected EBV B cell line (LCL) has abrogated CD1d surface expression, latently KSHV infected cell line (BCBL-1) does not have downregualted CD1d (Chung, et al. 2013; Sanchez, et al. 2005). As a result, we speculate that MHV-68 could downregulate CD1d expression during lytic replication between day 6 and 10 post-infection, and CD1d expression could return to normal beyond peak viral latency in the spleen on day 14 post-infection.

5.3 MHV-68 INFECTION IN C57BL/6 AND CD1d KO ADULT MICE

We did not observe severe MHV-68 infection and increased viral load in CD1d KO adult mice although adult mice received a higher infectious dose of virus during isoflurane anesthesia. This finding is in line with a study reporting that NK and NKT cells are not important to control MHV-68 infection (Usherwood, et al. 2005). We posit that mature C57BL/6 and CD1d KO mice had efficient antigen presentation in APCs and CD8+ T cell response, which lead to better control of MHV-68 infection than pups. It should also be noted that, in our experiment, the viral copy number in the spleen of infected adult mice was significantly lower than infected pups. We also observed increased spleen weight from infected pups although the difference in spleen weight between infected and uninfected pups was not statistically significant. We speculate that MHV-68 infection can induce splenomegaly in infected pups earlier than adult mice.
During acute phase of MHV-68 replication between day 6 to day 10 post-infection, the virus does not infect splenocytes. Since infected CD1d KO adult mice also had smaller size and lower viral copy number from the spleen compared to infected wild-type C57BL/6 adult mice, the decreased splenomegaly and viral copy number from infected adult mice were not due to lack of functional NKT cells. The results presented here suggest that NKT cells may be activated by CD1d independent manner, downregulated CD1d expression, or NKT cell independent control of MHV-68.

5.4 EARLY EBV INFECTION AND MHV-68 INFECTION

MHV-68 infection model in neonatal mice may not closely characterize early EBV infection in human. For example, early EBV infection dose is not studied well, and the initial site of viral replication occurs in oral epithelial cells while MHV-68 acute replication occurs in the lung (Hwang, et al. 2008). Furthermore, the use of inhalable anesthesia on mice can lead to different infection outcomes (Smee, et al. 2008). Smee et al. reported that larger inoculation volume during intranasal inoculation can lead to lower respiratory tract infection. Also, Milho et al. showed that use of isoflurane anesthesia during intranasal infection of MHV-68 can also cause lower respiratory tract infection (Milho, et al. 2009). As a result, it is possible that infected C57BL/6 and CD1dKO pups had equal and severe infection in the lower respiratory tract.
5.5 FUTURE DIRECTIONS

Although recent evidence favours NKT cell independent control of MHV-68 infection, it remains as speculation that NKT cell activation during MHV-68 infection may not require CD1d receptor. NKT cell maturation requires IL-15 (Gordy, et al. 2011). Since IL-15 is important for NKT cell maturation, it would be intriguing to infect IL-15 deficient pups with MHV-68 to determine CD1d independent control of MHV-68 infection. Furthermore, Flow cytometry with an NKT cell activation marker such as CD69 and NKT cell specific CD1d tetramer could shed further light on the activation and recruitment of NKT cells during acute MHV-68 infection in the lung.

Also, it is possible that NKT cells can play a regulatory role rather than controlling MHV-68 infection. Although C57BL/6 and CD1d KO pups and mice had equal viral load, it is possible that NKT cells can prevent expansion of CD8+ T cells. Ptaschinski et al. showed that splenocytes harvested on day 16 post-infection from infected pups had significant lower number of CD8+ T cells (Ptaschinski and Rochford 2008). Furthermore, there was an increase in lung weight from infected CD1d KO pups although the increase in lung weight was not statistically significant. We speculate that NKT cells can mediate anti-inflammatory response. It would be intriguing to confirm severe epithelial tissue cell damage from CD1d KO pups by immunohistochemistry.

Furthermore, expression of folkhead box P3 (FOXP3) expressing regulatory NKT cells from human PBMC and NKT cell clone have been described (Engelmann, et al. 2011). To obtain insight into regulatory NKT cells, it would be worthwhile to look at if activated NKT cells express FOXP3 and inhibit antiviral CD8 T' cell response in IM adult patients and
healthy EBV seropositive individuals. Although the regulatory role of NKT cells remains elusive, we speculate that uncontrolled expansion of CD8^+ T cells in IM patients is caused by lack of regulatory NKT cell.
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